AD_____

Award Number: W81XWH-10-1-0357

TITLE: Studying the role of eukaryotic translation initiation factor 4E (eIF4E) phosphorylation by MNK1/2 kinases in prostate cancer development and progression

PRINCIPAL INVESTIGATOR: Bruno Fonseca

CONTRACTING ORGANIZATION: McGill University

Montreal H3A 2T5

REPORT DATE: June 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2013	Annual Summary	1st June 2010 – 31 May 2013

4. TITLE AND SUBTITLE
Studying the role of eukaryotic translation initiation factor 4E (eIF4E) phosphorylation by MNK1/2 kinases in prostate cancer development and progression

5a. CONTRACT NUMBER
W81XWH-10-1-0357

5b. GRANT NUMBER W81XWH-10-1-0357

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)
Bruno Fonseca

E-Mail: bd.fonseca@mcgill.ca

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
McGill University
Montreal (H3A 1A3), Quebec,
Canada

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The mRNA cap-binding protein eukaryotic initiation factor 4E (eIF4E) plays a key role in cancer progression. We have recently shown (Furic et al., 2010) that phosphorylation of eIF4E at Ser209 by the MNK family of kinases promotes prostate cancer progression in a mouse model bearing a tissue-specific conditional PTEN deletion in prostatic epithelia. The mechanism whereby eIF4E phosphorylation promotes prostate cancer development remains unexplored. Genome-wide analysis studies by our group (Furic et al. 2010) had shown that phosphorylation of eIF4E regulated the protein levels of a large number of proteins linked to remodeling of the tissue surrounding the tumor. Of note, the levels of MMP3, a matrix metalloprotease required for epithelia-mesenchymal transition (EMT), is decreased in eIF4E S209A knockin mouse embryo fibroblasts. In a logical continuity of this finding, next we investigated whether eIF4E phosphorylation is a determinant factor in metastatic progression. The ability of eIF4E to promote metastasis in various mouse models and its association with poor prognosis in the clinic is well documented (De Benedetti et al., 2004; Nasr et al., 2013; Pettersson et al., 2011; Graff et al., 2009), but the mechanism by which phospho-eIF4E may promote metastasis is unknown. In this final report, we elucidate a mechanism by which eIF4E phosphorylation drives metastatic progression. Specifically, we show that phosphorylation of eIF4E promotes the translation of Snail2 and MMP3, both key factors in epithelia-mesenchymal transition during metastasis. Our most recent findings yield important information for a better understanding of how phosphorylation of the proto-oncogene eIF4E drives cancer progression.

15. SUBJECT TERMS
eIF4E phosphorylation, MNKs, MMP3, Snail2, EMT, metastasis.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

Final report

Table of contents ***Page number***

1. Introduction

The 5' cap mRNA binding protein, eIF4E, is a master regulator of the initiation step of protein synthesis. eIF4E binds to other translation initiation factors (eIF4G and eIF4A) to form the eIF4F complex which recruits the ribosomal machinery to the mRNA. eIF4E plays a key role in oncogenic transformation *in vitro* and phosphorylation at serine 209, the sole phospho-residue on eIF4E, promotes prostate cancer development in PTEN-deficient mouse models of prostate cancer (1). Genome-wide analysis studies from our lab have shown that translation of a subset of mRNAs is selectively decreased in mouse embryo fibroblasts deficient for eIF4E phosphorylation (eIF4E S209A knockin MEFs) (1). Notably, a large number of these mRNAs encode proliferation and pro-survival proteins such as BIRC2, as well as several paracrine factors involved in immune signaling/inflammation (chemokines CCL2, CCL7 and CCL9), and extracellular matrix rearrangements/angiogenesis (MMP3, MMP9 and VEGFC)(1). These proteins are known to mediate stromal-tumor interactions, suggesting that eIF4E phosphorylation may play an important role in the progression from benign prostate cancer to metastatic malignant prostate cancer by increasing the translation of specific mRNAs required for tumor migration and invasion. There is currently much interest (2,3) in the development therapeutic agents targeting eIF4E phosphorylation in malignant cancer. However, the importance of eIF4E phosphorylation in the context of metastatic progression remains unexplored. This is an important gap in our knowledge. The ability of eIF4E to promote mestastasis in various mouse models and its association with poor prognosis in certain cancers is well documented (4-7) but the mechanism whereby phospho-eIF4E may promote metastasis is unknown. In this final report, we elucidate a mechanism by which eIF4E phosphorylation promotes metastatic progression involving the upregulation of epithelial-mesenchymal-transition (EMT) and invasion promoting factors Snail2 and MMP3. EMT is the process by which epithelial cells normally bound to one another by tight junctions dissociate from each other, migrate and invade the extracellular matrix (ECM) (8). Invasion is driven by proteinases, notably the matrix metalloproteinases (MMPs), which digest the ECM allowing migration of the cancer cell into surrounding tissues (9). Overexpression of one such matrix metalloprotease, MMP3 is sufficient to induce EMT (10). In the present study, we show that phosphorylation of eIF4E upregulates the translation of MMP3 and Snail2 thus promoting tumor metastasis. Together, the findings presented herein implicate phosphorylation of eIF4E as a key event in the metastatic process and identify a mechanism of action, leading us to propose that therapeutic intervention on the MNK/eIF4E pathway may be a useful strategy for the prevention of tumor progression to the invasive and metastatic state in prostate cancer.

3. Body

Phosphorylation of eIF4E upregulates migration and invasion

Metastatic cancers typically display increased migration. Migration can be studied using a wound-healing assay (11). The speed at which cells close the wound, created by scratching reflects their ability to migrate. To evaluate the role of eIF4E phosphorylation in migration, MEFs isolated from eIF4E S209A KI mice and wildtype control littermates were transformed with C-MYC and H-RAS^{V12} as previously described (1). Migration was then measured for each cell type using the wound-healing assay. eIF4E S209A KI MEFs displayed decreased migration speed compared to wildtype controls, as determined by individual cell tracking by time-lapse microscopy (Fig. 1A). eIF4E S209A KI MEFs also displayed reduced invasion by as much as 3-fold in a transwell invasion assay (Fig. 1B). To further ascertain the role of eIF4E phosphorylation in invasion, we also examined the ability of transformed wildtype and KI MEFs to invade in a colony outgrowth assay (Fig. 1C). The latter assay allows for visual inspection of the ability of transformed cells to invade the surrounding matrigel. As shown in Fig. 1C, eIF4E wildtype MEFs formed highly branched colonies populated by invasive cells, while eIF4E S209 KI MEFs formed tight globular structures which failed to invade the matrigel (Fig. 1C), indicating that eIF4E phosphorylation contributes to the early steps of dissemination in metastasis. As expected, the MNK inhibitor CGP57380 reduced phosphorylation eIF4E and impaired invasion, but it did so to wildtype and eIF4E S209 KI MEFs alike (Fig. 1C). This can be explained by the non-specific nature of the CGP57380. This compound targets other kinases (e.g. Lck) within similar IC_{50} range of MNKs. It is also possible that other established MNK targets (e.g. Sprouty2, cPLA2 and hnRNPA1 (12-15) may also contribute to anti-invasive properties of CGP57380 downstream of MNKs.

Phosphorylation of eIF4E regulates the expression of Snail2/MMP3, known mediators of EMT

Cancer cells undergo architectural rearrangement associated with loss of polarity. This step is a pre-requisite for dissociation of cancer cells from the tumor tissue where they have originated and subsequent dissemination to distant organs/tissues (where they reattach and proliferate to give rise to metastases). Matrix metllaoproteases play a pivotal role in architectural rearrangement. Previous genome-wide analysis of the phospho-eIF4E translatome from our lab (1) revealed that MMP3 protein levels are downregulated in phosphorylation defective eIF4E S209A MEFs (Fig. 2A, B). Given that MMP3 levels are regulated by phospho-eIF4E, we next investigated whether inhibition of MNKs (the sole eIF4E kinase) with cercosporamide reduced serum-mediated production of MMP3 (Fig. 3A). Notably, cercosporamide reduced MMP3 protein to the levels found in eIF4E S209A MEFs. Similarly, Snail2 protein levels were also reduced upon treatment with cercosporamide. These results corroborate our earlier published finding that phospho-eIF4E regulates the levels of key proteins involved in the control of metastasis (1).

Phosphorylation of eIF4E upregulates translation of Snail2 and MMP3 to promote invasion

Our observation that MMP3 and Snail2 protein levels are reduced in eIF4E S209A KI MEFs prompted us to postulate that perhaps phospho-eIF4E regulates the translation of MMP3 and Snail2. To investigate this possibility, we performed polysome profile analysis on wildtype and eIF4E S209A MEFs. As expected and previously reported, there were no gross changes in global protein synthesis, noted by the similar polysomal profiles (Fig. 3B). Consistent with this, the distribution of housekeeping mRNAs such as beta-actin also remained unchanged. There was, however, a redistribution of the transcripts encoding MMP3 and Snail2 from polysomal (in wildtype) to subpolysomal (in eIF4E S209A KI) fractions, indicating that these mRNAs are subject to translational regulation by phosphorylated eIF4E (Fig. 3B). Lastly, we investigated whether induction phospho-eIF4E-regulated mRNAs could promote metastasis. To this end, we restored expression of MMP3 in eIF4E S209A MEFs by overexpressing either wildtype eIF4E or MMP3 lacking its regulatory 5' and 3'UTRs (Fig. 3D). Overexpression of either wildtype eIF4E or MMP3 coding sequence rescued the invasion phenotype. In contrast, expression of the phospho-defective mutant of eIF4E (S209A) did not restore invasion, demonstrating that phosphorylated eIF4E promotes invasion and metastasis through translational upregulation of MMP3 (Fig. 3E).

3. Key research accomplishments

a. Demonstrated that phosphorylation of eIF4E is important to cancer metastasis.

b. Identified Snail2 and MMP3, known important mediators of EMT, as two novel translational targets of phosphorylated eIF4E,

c. Demonstrated that translational upregulation of MMP3 by phosphorylated eIF4E is required for migration and invasion during metastasis.

4. Reportable outcomes

None declared.

5. Conclusion

There is currently a high level of interest in the development of pharmacological inhibitors of MNKs, and consequently of eIF4E phosphorylation, some of which (*e.g.* cercosporamide) have already been shown to suppress neoplastic growth of cancer cells in animal models (2). There is, however, a void of information on whether eIF4E phosphorylation is important in metastasis, and thus whether MNK inhibitors would work in the treatment of prostate cancer post-dissemination. Our study demonstrates that phosphorylation of eIF4E is key for the transition to malignancy. We further elucidate the mechanism whereby phosphorylation of eIF4E may contribute to a tumor's full metastatic potential. Our latest findings corroborate and provide logical continuity to earlier work from our laboratory by Furic and colleagues (1). We anticipate that our findings on the lack of metastatic potential of phosphorylation-deficient eIF4E tumors will be of significant interest to the scientific community. Moreover, we firmly believe that they will provide invaluable information for the design of efficient therapeutic anti-cancer agents with potential use in prostate cancer treatment.

6. References

1. Furic, L., Rong, L., Larsson, O., Koumakpayi, I. H., Yoshida, K., Brueschke, A., Petroulakis, E., Robichaud, N., Pollak, M., Gaboury, L. A., Pandolfi, P. P., Saad, F., and Sonenberg, N. (2010) *Proc Natl Acad Sci U S A* **107**, 14134-14139
2. Lee, T., and Pelletier, J. (2012) *Future Med Chem* **4**, 19-31
3. Jia, Y., Polunovsky, V., Bitterman, P. B., and Wagner, C. R. (2012) *Med Res Rev* **32**, 786-814
4. De Benedetti, A., and Graff, J. R. (2004) *Oncogene* **23**, 3189-3199
5. Nasr, Z., Robert, F., Porco, J. A., Jr., Muller, W. J., and Pelletier, J. (2013) *Oncogene* **32**, 861-871
6. Pettersson, F., Yau, C., Dobocan, M. C., Culjkovic-Kraljacic, B., Retrouvey, H., Puckett, R., Flores, L. M., Krop, I. E., Rousseau, C., Cocolakis, E., Borden, K. L., Benz, C. C., and Miller, W. H., Jr. (2011) *Clin Cancer Res* **17**, 2874-2884
7. Graff, J. R., Konicek, B. W., Lynch, R. L., Dumstorf, C. A., Dowless, M. S., McNulty, A. M., Parsons, S. H., Brail, L. H., Colligan, B. M., Koop, J. W., Hurst, B. M., Deddens, J. A., Neubauer, B. L., Stancato, L. F., Carter, H. W., Douglass, L. E., and Carter, J. H. (2009) *Cancer Res* **69**, 3866-3873
8. Kalluri, R. (2009) *J Clin Invest* **119**, 1417-1419
9. Friedl, P., and Alexander, S. (2011) *Cell* **147**, 992-1009
10. Lochter, A., Galosy, S., Muschler, J., Freedman, N., Werb, Z., and Bissell, M. J. (1997) *J Cell Biol* **139**, 1861-1872
11. Rodriguez, L. G., Wu, X., and Guan, J. L. (2005) *Methods Mol Biol* **294**, 23-29
12. DaSilva, J., Xu, L., Kim, H. J., Miller, W. T., and Bar-Sagi, D. (2006) *Mol Cell Biol* **26**, 1898-1907
13. Hefner, Y., Borsch-Haubold, A. G., Murakami, M., Wilde, J. I., Pasquet, S., Schieltz, D., Ghomashchi, F., Yates, J. R., 3rd, Armstrong, C. G., Paterson, A., Cohen, P., Fukunaga, R., Hunter, T., Kudo, I., Watson, S. P., and Gelb, M. H. (2000) *J Biol Chem* **275**, 37542-37551
14. Guil, S., Long, J. C., and Caceres, J. F. (2006) *Mol Cell Biol* **26**, 5744-5758
15. Buxade, M., Parra, J. L., Rousseau, S., Shpiro, N., Marquez, R., Morrice, N., Bain, J., Espel, E., and Proud, C. G. (2005) *Immunity* **23**, 177-189

7. Appendix

No appendix is included

8. Support Data

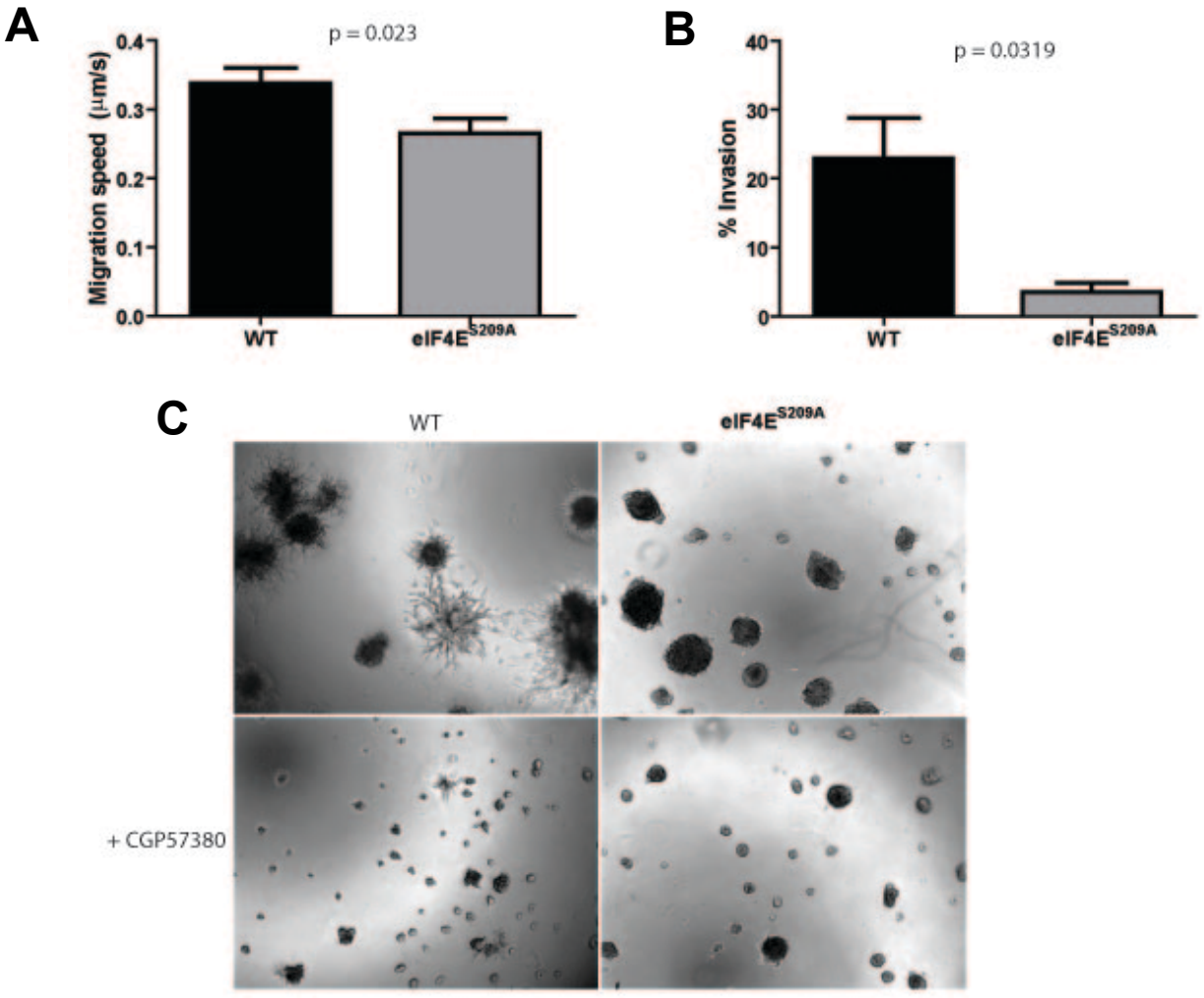

Fig. 1. eIF4E phosphorylation promotes migration and invasion. MEFs isolated from wildtype and eIF4E S209A knockin (KI) mice were transformed with c-MYC and H-RAS^{V12}. Migration speed and invasive potential was determined by wound healing assay (a), transwell invasion assay (b) and colony outgrowth assay (c), respectively. Representative micrographs of eIF4E wildtype (c-MYC/H-RAS ^{V12}) and eIF4E S209A KI (c-MYC/H-RAS^{V12}) colonies in colony outgrowth assay are shown.

Fig. 1

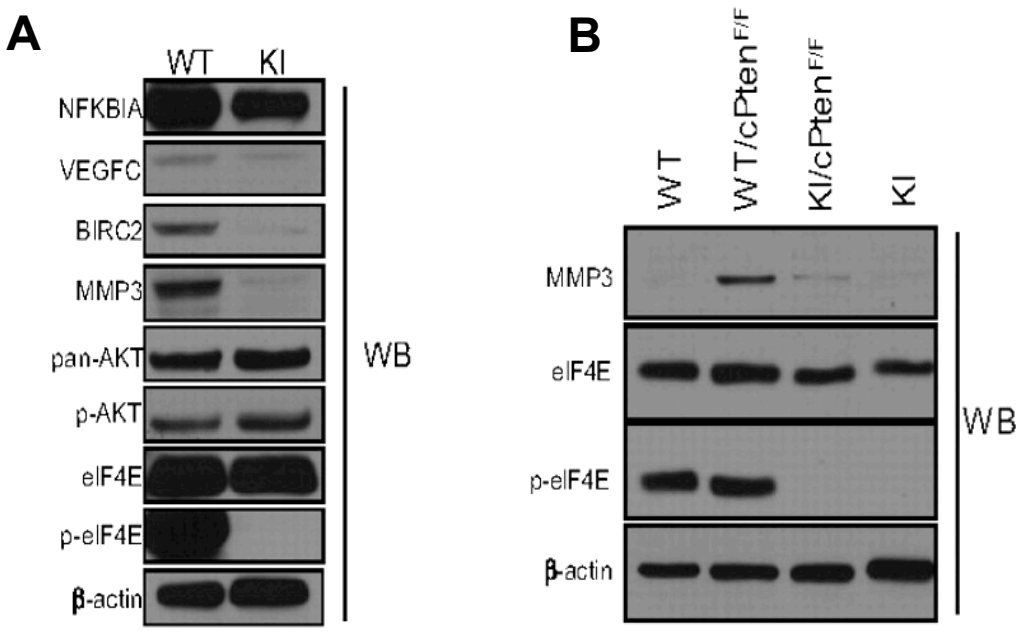

Fig. 2. Phosphorylation of eIF4E at Ser209 is important for MMP synthesis in prostate cancer. (a) Serum starved WT and KI MEFs were serum-stimulated for 2 h and cell lysates were resolved by SDS-PAGE followed by western blotting with the indicated antibodies. (b) DLP extracts were resolved by SDS/PAGE followed by Western blotting performed with the indicated antibodies. (These data were extracted from Furic *et al.*, 2010 for illustration purposes).

Fig. 2

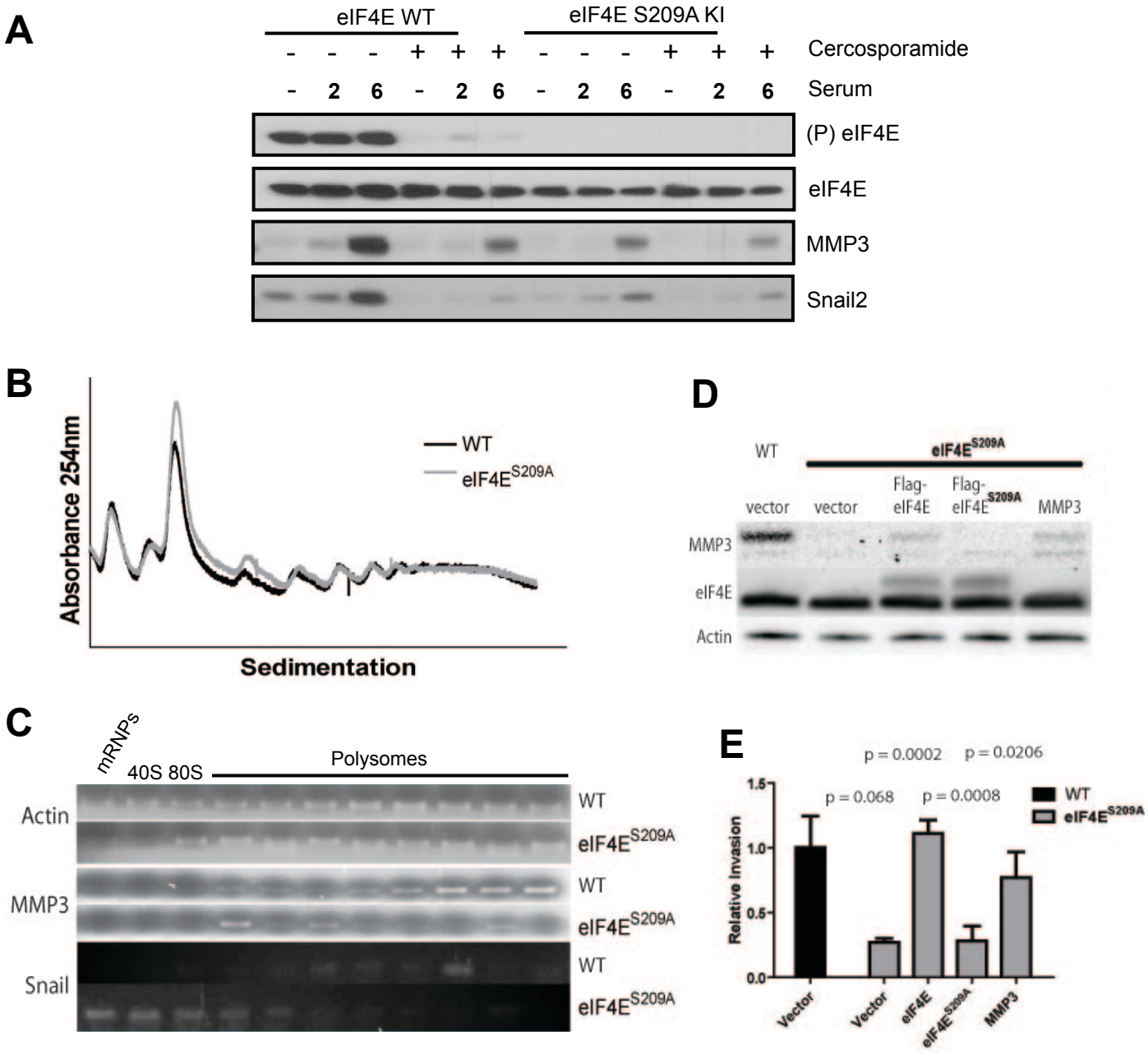

Fig. 3. MNKs regulate MMP3 and Snail2 protein levels via phospho-eIF4E-dependent translation. (a) Wildtype and eIF4E S209A KI MEFs starved of serum overnight and subsequently stimulated with 10% (v/v) fetal bovine serum for the indicated times. Where indicated cells were treated with the MNK inhibitor cercosporamide prior to stimulation with serum. Cell extracts were analyzed by SDS-PAGE/Western blotting with indicated antibodies. (b) Polysome profile and (c) semi-quantitative RT-PCR analysis of mRNAs in subpolysomal and polysomal fractions from wildtype and eIF4E S209A MEFs. (d) Overexpression of phospho-eIF4E promotes MMP3 expression as assessed by Western blot. (e) Cell invasion is upregulated by expression of either phospho-eIF4E or MMP3 as determined in a transwell invasion assay.

Fig. 3